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OPTIMIZATION OF FERMENTATION MEDIUM AND FERMENTATION CONDITIONS OF BIOCONTROL STRAIN F11

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Abstract

Peanut web blotch is one of the more prevalent and rapidly spreading diseases of peanut. In this study, soil samples were collected from different crops and vegetable rhizosphere soils in different regions, and biocontrol fungus F11 was isolated and screened. The strain had a good inhibitory effect on *Didymella arachidicola*, and it was identified as *Talaromyces sayulitensis* by morphological identification and molecular biology. The fermentation medium and fermentation conditions were optimized by single factor test, orthogonal test and response surface test. The results showed that the optimum fermentation medium was corn gluten meal 24.33 g·L^{-1}, diammonium hydrogenphosphate 2.56 g·L^{-1}, and $CaCO_3$ 1.52 g·L^{-1}. The model was established by Design Expert software, and the bacteriostatic rate under the above conditions was predicted to be 87.92%. The actual inhibition rate obtained after verification was 87.65%, which was close to the predicted value. Compared with 63.95% before optimization, it increased by 23.70%. The optimum fermentation conditions were as follows : liquid volume was 125 mL, temperature was 30°C, and fermentation time was 7 d.

Key words: *Didymella arachidicola*; Biological defense fungi; *Talaromyces sayulitensis*; Response surface methodology.

Introduction

Peanut web blotch mainly occurs in the middle and late stages of peanut growth, mainly affecting leaves, and can also harm petioles and stems in severe cases. The disease has fast incidence, fast spread and serious harm (Fan *et al.*, 2022). Peanut web blotch disease, also known as brown streak disease. In 1972, the disease was first discovered in Texas, the United States, and then many countries reported that the disease occurred. In 1982, peanut web blotch was reported in the main peanut producing areas of Shandong and Liaoning provinces in China. Since then, the disease has also occurred in Shaanxi, Henan and other provinces. At present, the disease is widespread in the main peanut producing areas in Anyang, Henan Province, which poses a serious threat to the safety and quality of peanut production. In the main peanut producing areas of Henan Province, peanut web blotch generally began to occur in the flowering stage. August to September is the peak period of the disease, which mainly harms the leaves, but also harms the stems and petioles. Peanut web blotch can lead to a large number of leaves falling in the late growth stage of peanut, which seriously affects the yield of peanut. The yield is reduced by 10% ~ 20%, and the serious is more than 30%. The epidemic year can cause 20% ~ 40% yield loss (Qi & Lu, 2023; Xu *et al*., 1995). In previous studies, Chen *et al*., divided the pathogen of peanut web blotch into *Didymella* according to phylogenetic and morphological observation, and this paper uses the latest name *Didymella arachidicola* (Li *et al*., 2018).

At present, some research has been done in the field of biological control at home and abroad. The control of fungal diseases in most crops generally relies on chemical control. The use of fungicides not only increases production costs, but also brings environmental pollution and pesticide residues in agricultural products. Therefore, in recent years, it is more meaningful to screen biocontrol strains and develop fungicides to control plant diseases. In this experiment, by screening biocontrol strains and optimizing the culture conditions of the strains, the culture efficiency of the strains can be improved, the growth rate and activity of the strains can be improved, and the resistance and tolerance of the strains can be improved. Therefore, the produced bacterial liquid is better and the research is more meaningful. The results showed that the compound effect of biocontrol agent was better than that of single biocontrol agent. This experiment provided theoretical and practical basis for the production of biocontrol agent against peanut web blotch. It paves the way for the subsequent production of compound biocontrol agents to prevent diseases (Zhu *et al*., 2017; Zang *et al*., 2021; Yang *et al*., 2023).

Materials and Methods

Test strain: *Didymella arachidicola*, *Talaromyces sayulitensis*.

Test medium: Potato Dextrose Agar (PDA) medium: Potato 200 g·L^{-1}, Dextrose 20 g·L^{-1}, Agar 15 g·L^{-1}.

Potato dextrose (PD) medium: Potato 200 g·L^{-1}, Glucose 20 g·L^{-1}.

Carbon source test medium: Glucose, Sucrose, Soluble starch, Corn gluten meal, Mannitol, Dextrin, Maltose, Lactose, Fructose.

Nitrogen source test medium: Beef paste, Peptone, Tryptone, Potassium nitrate, Ammonium chloride, Soybean meal, Fine bran, Diammonium hydrogen phosphate, Urea, Yeast leaching powder.

Inorganic salt test medium: $CuSO_4·5H_2O$, $ZnSO_4·7H_2O$, $MgSO_4·7H_2O$, $FeSO_4·7H_2O$, $K_2HPO_4·3H_2O$, $CaCO_3$, KH_2PO_4, NaCl.

pH test medium: the pH of PD medium was adjusted to 5.0, 6.0, 7.0, 8.0 and 9.0 with 1.0 mol·L^{-1} HCl and 1.0 mol·L^{-1} NaOH, respectively.

Screening of optimal components in fermentation media: Single factor test: Two 8 mm F11 biocontrol fungal cakes were inoculated in 35 carbon sources, nitrogen sources, inorganic salts and pH test media, respectively. Three replicates were set and cultured at 25℃and 175 rpm for 7 days. Different fermentation broths were mixed with PDA medium at a ratio of 1:5 and placed in a petri dish for 30 min, and then inoculated with *Didymella arachidicola*. Three replicates were set up, and sterile water and PDA medium were mixed at a ratio of 1:5 as the control group. They were cultured in an incubator at 25℃, and the colony diameter was measured when the control colony grew completely. SPSS software was used for significance analysis.

Orthogonal test: carbon source, nitrogen source, inorganic salt and pH were 4 factors, and the optimal 3 components of each factor obtained from single factor were 3 levels. The optimal combination of components was obtained by L9 (34) orthogonal test design (Table 1).

Optimization of the addition amount of fermentation medium components: Based on the optimal component combination obtained by orthogonal test, the addition amount of carbon source, nitrogen source and inorganic salt was optimized by response surface test, and a set of optimal liquid medium suitable for strain F11 fermentation was screened.

Using the magnitude of bacterial inhibition as a response index, the one-way rotation method was used to examine sequentially the corn gluten meal additions (15.00 g·L^{-1}, 20.00 g·L^{-1}, 25.00 g·L^{-1}, 30.00 g·L^{-1}, 35.00 g·L^{-1}), diammonium hydrogen phosphate additions (1.50 g·L^{-1}, 2.00 g·L^{-1}, 2.50 g·L^{-1}, 3.00 g·L^{-1}, 3.50 g·L^{-1}), and $CaCO_3$ addition (1.00 g·L^{-1}, 1.25 g·L^{-1}, 1.50 g·L^{-1}, 1.75 g·L^{-1}, 2.00 g·L^{-1}) on the inhibition rate. The initial conditions of fermentation were temperature 25°C, rotational speed of 175 rmp, time of 7 d, and pH 7.0.

According to the results of single factor test, the range of response surface test parameters for the addition of fermentation medium components of strain F11 was determined. The Box-Behnken experimental design and analysis method were used to carry out the response surface test of 3 factors and 3 levels, and the optimal process parameters of strain F11 fermentation were obtained. Analyze and process the data obtained by Design Expert 8.0.6 (Zhou *et al*., 2024; Chen *et al*., 2024; Zhao *et al*., 2024; Xue *et al*., 2024).

Fermentation condition optimization: The loading volume was set at 50 mL, 75 mL, 100 mL, 125 mL and 150 mL, the fermentation time was 4 d, 5 d, 6 d, 7 d and 8 d, and the fermentation temperatures were 15℃, 20℃, 25℃, 30℃ and 35℃, and the bacterial inhibition rates of the fermentation broths were determined to select the most suitable fermentation conditions (Zhang *et al*., 2023; Zhang *et al*., 2024; Sun *et al*., 2024a and Sun *et al*., 2024b).

Results and Analysis

Optimization of fermentation medium composition

Single-factor test: After the biocontrol strain F11 was fermented with different carbon sources, nitrogen sources, inorganic salts and pH media, the antibacterial effect of the fermentation filtrate was more obvious.

Screening of carbon sources: Firstly, 9 carbon sources and no carbon source were screened out. The results showed that the three with the lowest bacteriostatic rate were: Fructose (1.77%) < Maltose (2.07%) < Mannitol (2.31%); the three kinds with the highest inhibition rate were : Corn gluten meal (62.36%) > Lactose (49.61%) > No carbon source (37.13%). After fermentation with medium supplemented with corn gluten meal, lactose or no carbon source, the antibacterial effect was significantly better than other carbon sources at the $P = 0.05$ level (Fig. 1).

Screening of nitrogen sources: 10 kinds of nitrogen sources and no nitrogen sources were screened out. The three kinds of nitrogen sources with the lowest inhibition rate were : No nitrogen source (2.64%) < Peptone (5.93%) < Tryptone (7.57%). The three kinds with the highest inhibition rate were Urea (63.67%) > Ammonium chloride (62.61%) > Diammonium phosphate (62.88%). After fermentation with the added medium, the bacteriostatic rate was significantly higher than that without adding nitrogen source (Fig. 2).

Screening of inorganic salts: Eight kinds of inorganic salts and non-added inorganic salts were screened. According to the graph, the three with the lowest bacteriostatic rate were: Non-added inorganic salts (6.46%) < NaCl (11.31%) < KH_2PO_4 (14.15%); the three kinds with the highest inhibition rate were $ZnSO_4$ $7H_2O$ (74.52%) > $CuSO_4$ $5H_2O$ (74.49%) > $CaCO_3$ (74.45%) (Fig. 3).

In the pH screening test: When the pH was 5,6 and 7, the bacteriostatic effect of fermentation was the best (Fig. 4).

Table 1. Factors and levels of orthogonal design.

Level	Factor			
	A Carbon source	B Nitrogen source	C Inorganic salt	D pH
1	Corn gluten meal	Urea	$ZnSO_4·7H_2O$	5
2	Lactose	Ammonium chloride	$CaCO_3$	6
3	Not add	Diammonium hydrogen phosphate	$CuSO_4·5H_2O$	7

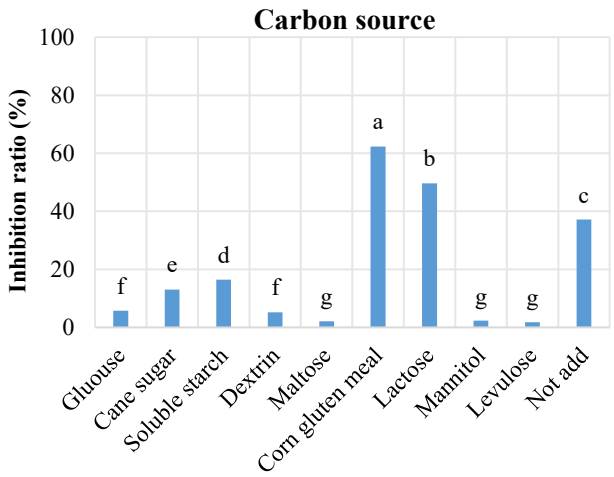

Fig. 1. Inhibition ratio of different carbon source medium fermentation. Note: Different lowercase letters indicate significant differences at the *P*=0.05 level.

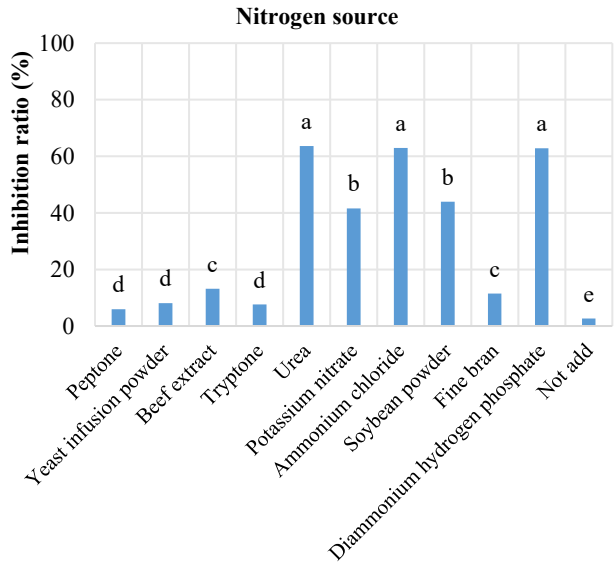

Fig. 2. Inhibition ratio of different nitrogen source medium fermentation.

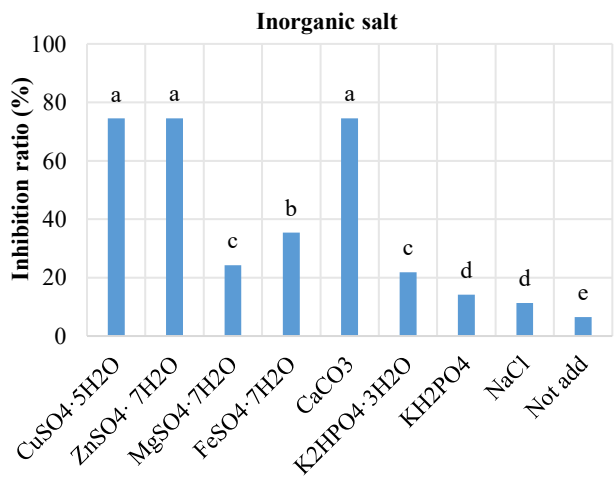

Fig. 3. Inhibition ratio of different inorganic salt medium fermentation.

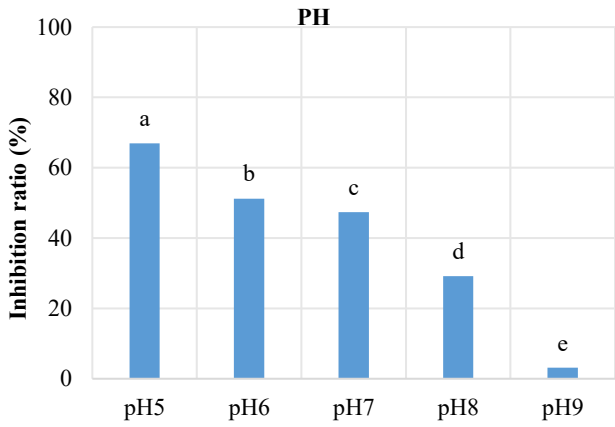

Fig. 4. Inhibition ratio of different pH medium fermentation

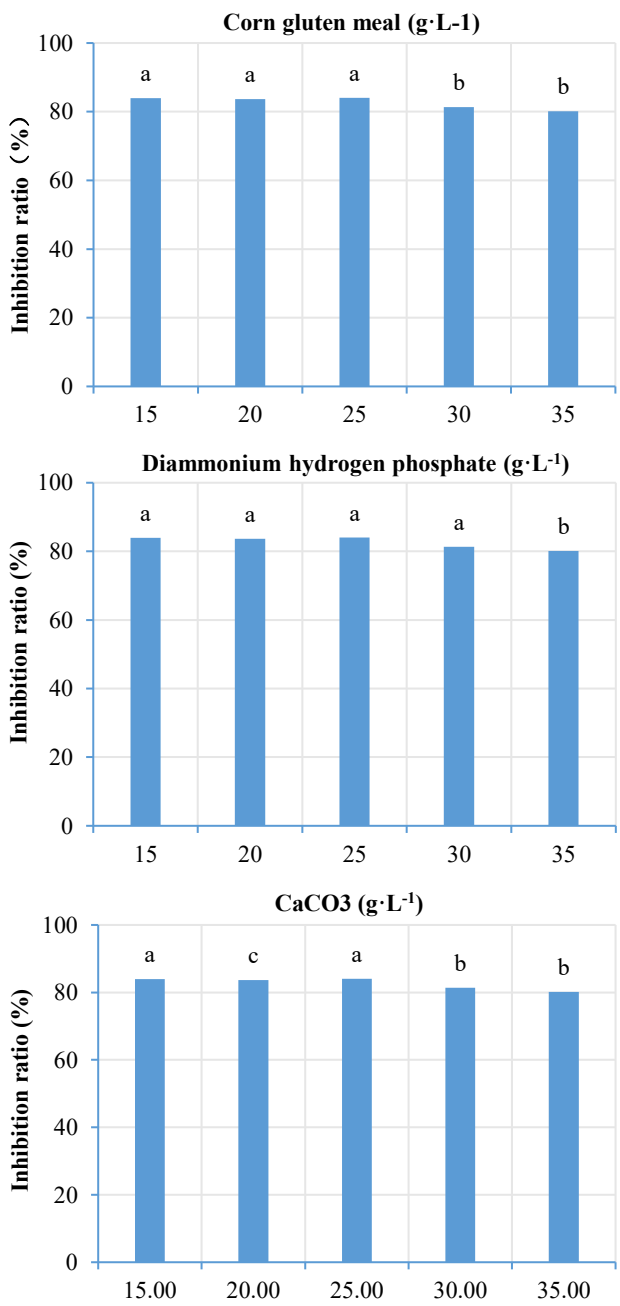

Fig. 5. Effect of supplemental levels of corn gluten meal, diammonium hydrogen phosphate and $CaCO_3$ on inhibition rate.

Table 2. The intuitive analysis of the orthogonal experiments.

Test number	Factor				Inhibition
	A	B	C	D	rate(%)
1 ($A_1B_1C_1D_1$)	Corn gluten meal	Urea	$ZnSO_4·7H_2O$	5	62.84±0.6951[g]
2 ($A_1B_2C_3D_2$)	Corn gluten meal	Ammonium chloride	$CuSO_4·5H_2O$	6	82.21±5.1074[c]
3 ($A_1B_3C_2D_3$)	Corn gluten meal	Diammonium hydrogen phosphate	$CaCO_3$	7	86.92±0.7720[a]
4 ($A_2B_1C_3D_3$)	Lactose	Urea	$CuSO_4·5H_2O$	7	44.28±0.5154[h]
5 ($A_2B_2C_2D_1$)	Lactose	Ammonium chloride	$CaCO_3$	5	72.95±0.4050[f]
6 ($A_2B_3C_1D_2$)	Lactose	Diammonium hydrogen phosphate	$ZnSO_4·7H_2O$	6	84.45±3.9010[b]
7 ($A_3B_1C_2D_2$)	Not add	Urea	$CaCO_3$	6	39.29±5.6308[i]
8 ($A_3B_2C_1D_3$)	Not add	Ammonium chloride	$ZnSO_4·7H_2O$	7	74.27±1.8955[e]
9 ($A_3B_3C_3D_1$)	Not add	Diammonium hydrogen phosphate	$CuSO_4·5H_2O$	5	80.73±1.8494[d]

Note: Different lowercase letters indicate significant differences at the P=0.05 level

Table 3. Range analysis results of various factors.

Index	Factor			
	A	B	C	D
K_1	231.97	146.41	221.56	216.52
K_2	201.68	229.43	199.16	205.47
K_3	194.29	252.10	207.22	205.95
k_1	77.32	48.80	73.85	72.17
k_2	67.23	76.48	66.39	68.49
k_3	64.76	84.03	69.07	68.65
R	12.56	35.23	7.46	3.68

Note: K_1, K_2 and K_3 are the sum of all levels of each factor; k_1, k_2 and k_3 are the average values of the level of each factor; R value of are the range of k_1, k_2 and k_3

Analysis of orthogonal test results of inhibition rate of fermentation broth: It can be seen from the orthogonal test data and the range analysis results that the maximum range (R) of factor B is 35.23, which is the primary factor affecting the inhibition rate. The range values of factor A and factor C were 12.56 and 7.46, respectively. The range of factor D is only 3.68. The importance of the influence on the inhibition rate was nitrogen source, carbon source, inorganic salt and pH. The results showed that the optimal combination of fermentation medium components was $A_1B_3C_2D_3$, that is, when the carbon source was corn gluten meal, the nitrogen source was diammonium hydrogen phosphate, the inorganic salt was $CaCO_3$, and the pH was 7, the bacteriostatic rate was the highest, and the bacteriostatic rate was 86.92 % (Tables 2 and 3).

Optimization of fermentation medium composition addition amount

Single-factor test: It can be seen from Fig. 5 that the inhibitory effect of the strain on *Didymella arachidicola* was significantly different after fermentation in the medium supplemented with 1.00 g·L^{-1} ~ 2.00 g·L^{-1} $CaCO_3$. When the addition amount of $CaCO_3$ was 1.50 g·L^{-1}, the inhibition rate of *Didymella arachidicola* was the highest, which was 85.85%. The inhibitory effect of 15.00 g·L^{-1} ~ 35.00 g·L^{-1} corn gluten meal on the fermentation filtrate was slightly different. When the addition amount of corn gluten meal was 25.00 g·L^{-1}, the inhibition rate of the pathogen was the highest, which was 84.01%. When the addition amount of diammonium phosphate was 1.50 g·L^{-1} ~ 3.50 g·L^{-1}, there was no significant difference in the bacteriostatic rate of the fermentation broth, especially between 1.50 g·L^{-1} ~ 3.00 g·L^{-1}, and the bacteriostatic rate was between 74.13% ~ 76.54%. However, it decreased slightly at 3.50 g·L^{-1}, and the inhibition rate was 72.26%. Therefore, the optimum addition amount of corn gluten meal, diammonium phosphate and $CaCO_3$ in liquid fermentation medium was 25.00 g·L^{-1}, 2.50 g·L^{-1} and 1.50 g·L^{-1} , respectively.

Response surface optimization test: The establishment of the model and the significance test, with the inhibition rate (Y) as the response value, the amount of corn gluten meal added (X_1), the amount of diammonium phosphate added (X_2), and the amount of $CaCO_3$ added (X_3) as the investigation factors, with −1, 0, and 1 as the representative variable levels (Table 4), Design Expert 8.0.6 was used to design a three-factor three-level response surface optimization test to determine the optimal addition combination. The response surface optimization test data are shown in Table 5.

Using Design expert V8.0.6.1 software to fit the scores in Table 5, the regression equation was obtained: $Y= 87.80 -0.86\ X_1+0.85\ X_2+0.34\ X_3+0.76\ X_1X_2-0.015\ X_1X_3 +0.33\ X_2X_3-2.85\ X_1^2-3.17\ X_2^2-2.89\ X_3^2$. It can be concluded from the equation that in this model, the inhibition rate of fermentation broth is greatly affected by the amount of $CaCO_3$ added, followed by the amount of corn gluten meal added, and the amount of diammonium phosphate added has the least effect.

Analysis of variance and significance of the inhibition rate score (Table 6) showed that the F value of the established model was 6.88, and P was 0.0094, indicating that the model was significant. The F value of the mismatch term is 0.33, P (=0.8028) > 0.05, which means that the mismatch term is not significant relative to the error term, indicating that the model has high reliability. R2=0.8984, adjusted R2Adj=0.7678 showed that the model error was small and the fitting degree was high. The difference between predicted R2Pred = 0.5474 and adjusted R2Adj is also within a reasonable range. Signal-to-noise ratio (Adeq Precision) = 7.359 > 4 also confirms that the model is ideal.

Response surface curve analysis: The response surface curve and contour line of the interaction of corn gluten meal, diammonium hydrogen phosphate and $CaCO_3$ addition on the inhibition rate (Fig. 6).

Table 4. Variables and level of Box-Behnken design.

Level	Factor		
	X_1 Addition amount of corn gluten meal ($g \cdot L^{-1}$)	X_2 Addition amount of diammonium hydrogen phosphate ($g \cdot L^{-1}$)	X_3 Addition amount of $CaCO_3$ ($g \cdot L^{-1}$)
-1	20	2.00	1.25
0	25	2.50	1.50
1	30	3.00	1.75

Table 5. The results of response surface optimization test for strain F11 fermentation.

Code	Factors and levels			Inhibition rate(%)
	X_1 ($g \cdot L^{-1}$)	X_2 ($g \cdot L^{-1}$)	X_3 ($g \cdot L^{-1}$)	
1	-1	-1	0	82.48
2	1	-1	0	78.43
3	-1	1	0	83.60
4	1	1	0	82.60
5	-1	0	-1	82.27
6	1	0	-1	81.40
7	-1	0	1	82.75
8	1	0	1	81.82
9	0	-1	-1	81.24
10	0	1	-1	81.34
11	0	-1	1	81.47
12	0	1	1	82.91
13	0	0	0	87.65
14	0	0	0	86.80
15	0	0	0	86.38
16	0	0	0	87.32
17	0	0	0	90.87

In Fig. 6, A and B are represented as the interaction between the addition of corn gluten meal and diammonium hydrogen phosphate when the addition of $CaCO_3$ is at the central level of 1.50 $g \cdot L^{-1}$, indicating that the inhibition rate increases first and then decreases with the increase of the addition of corn gluten meal and diammonium hydrogen phosphate. The C and D in Fig. 6 are represented by the interaction between the amount of corn gluten meal and the amount of $CaCO_3$ added when the amount of diammonium hydrogen phosphate is at the central level of 2.5 $g \cdot L^{-1}$, indicating that the inhibition rate increases first and then decreases with the increase of the amount of corn gluten meal and the amount of $CaCO_3$ added. The E and F in Fig. 6 are represented by the interaction between the amount of diammonium hydrogen phosphate and the amount of $CaCO_3$ added when the amount of corn gluten meal is at the central level of 25 $g \cdot L^{-1}$. The results show that the inhibition rate increases first and then decreases with the increase of the amount of diammonium hydrogen phosphate and the amount of $CaCO_3$ added.

The software was used to predict the maximum response value. The model of each factor combination of corn gluten meal was 24.33 $g \cdot L^{-1}$, diammonium hydrogen phosphate was 2.56 $g \cdot L^{-1}$, $CaCO_3$ was 1.52 $g \cdot L^{-1}$, the inhibition rate was 87.65%. According to the optimal conditions given by the model, the experiment was carried out, and the bacteriostatic rate was 87.92%, which was close to the predicted value, which proved that the model was effective. It is 23.70% higher than that before optimization (Fig. 7).

Fermentation condition optimization: The effects of liquid volume, fermentation time and fermentation temperature on the bacteriostatic effect of *Didymella arachidicola* were studied. The results showed that the bacteriostatic rate was the highest (82.75%) when the medium volume was 125 mL (250 mL conical flask), which was less different from 50 mL, 75 mL and 150 mL, but significantly better than 100 mL. When the fermentation time was 7 days, the bacteriostatic rate was the highest (82.37%), which was slightly different from that of 6 days, but significantly better than that of 4,5 and 8 days (Fig. 8). When the fermentation temperature was 30°C, the antibacterial rate was the highest, which was 85.10%, which was significantly better than other temperatures. Therefore, the optimum fermentation conditions were as follows: fermentation at 30°C for 7 d in 125 mL (250 mL conical flask) medium (Tian *et al*., 2022; Du *et al*., 2017; Liang *et al*., 2013; Gu *et al*., 2022; Liu *et al*., 2023; Chen *et al*., 2021; Zhou *et al*., 2020; Liu *et al*., 2006; Chen *et al*., 2020).

Table 6. Variance analysis of response surface experiments results.

Source	Sum of squares	df	Mean square	F Value	*P*-value Prob > F	Sig.
Model	140.36	9	15.60	6.88	0.0094	##
X_1	5.87	1	5.87	2.59	0.1518	
X_2	5.83	1	5.83	2.57	0.1528	
X_3	0.91	1	0.91	0.40	0.5463	
X_1X_2	2.33	1	2.33	1.03	0.3449	
X_1X_3	9.000E-004	1	9.000E-004	3.969E-004	0.9847	
X_2X_3	0.45	1	0.45	0.20	0.6698	
X_1^2	34.28	1	34.28	15.12	0.0060	##
X_2^2	42.40	1	42.40	18.70	0.0035	##
X_3^2	35.18	1	35.18	15.52	0.0056	##
Residual	15.87	7	2.27			
Lack of Fit	3.18	3	1.06	0.33	0.8028	
Pure Error	12.69	4	3.17			
Cor Total	156.23	16				

Note: "#"indicated significant impact on the results ($0.01<p<0.05$); "##"indicated that the impact on the results was extremely significant ($p<0.01$)

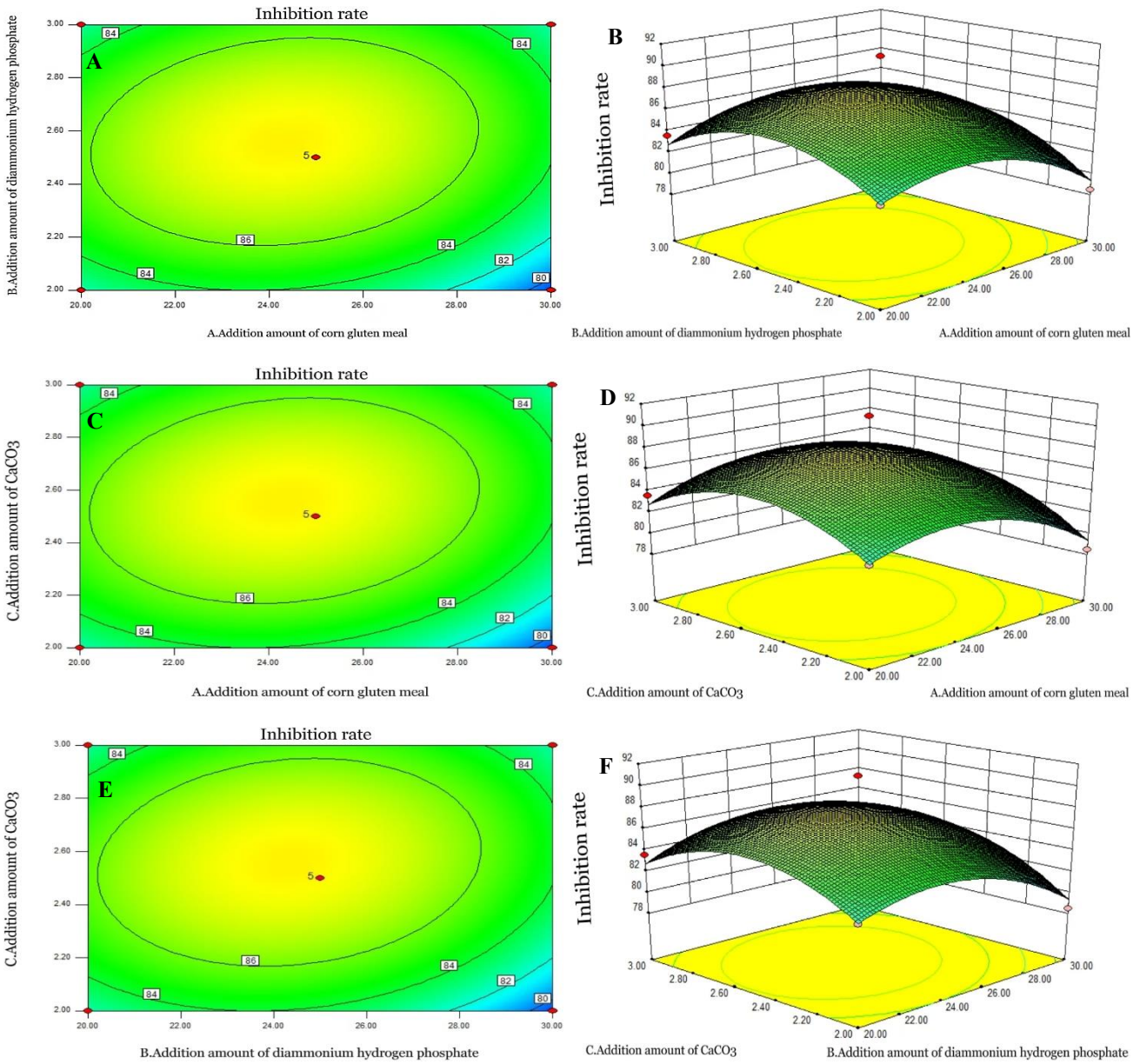

Fig. 6. Contour and 3D surface maps of interactions between factors.

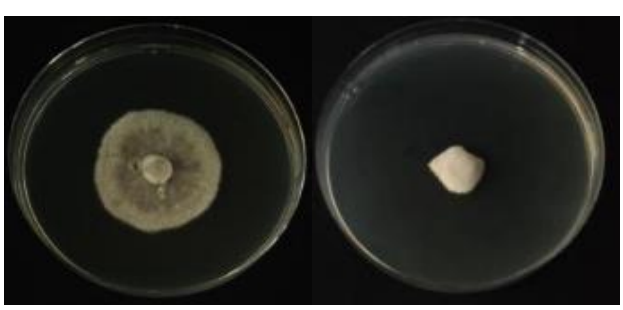

Fig. 7. Comparison of bacteriostatic effect under initial conditions (left) and optimized (right).

Discussions

Peanut web blotch is common in China. When the disease occurs more seriously, it causes a decrease in peanut yield and reduces the cost of peanuts. However, the current research on the control of fungal diseases relies more on chemical control, and chemical control has some limitations. It will destroy the environment and cause pesticide residues in agricultural products. As a better method of plant disease control, biological control should be strengthened in the research of biological control.

In the process of microbial fermentation, bacterial biomass is closely related to fermentation conditions. Generally, biocontrol bacteria can grow on the basic medium, but their growth status is closely related to the types of carbon and nitrogen sources. The appropriate type, proportion and concentration of carbon and nitrogen sources are more conducive to the growth of strains. The optimization of fermentation conditions mainly includes two parts: the optimization of medium composition including the selection of carbon source, nitrogen source, inorganic salt and pH and the determination of concentration; the optimization of culture conditions: inoculation amount, initial pH, culture temperature, shaking speed, culture time, etc. The growth of the strain is

not only related to the type of carbon source and nitrogen source but also affected by its ratio. By optimizing the type and ratio of carbon source, nitrogen source and inorganic salt, screening the best growth medium for the strain is an important measure to improve the antibacterial activity and fermentation efficiency. At present, the optimization methods mainly include single factor method, orthogonal test method and response surface method. The single factor method is simple and suitable for the consideration of a single variable; the orthogonal test method and the response surface method can analyze the interaction between single factors more comprehensively under the limited test amount. Most studies combine single factor method with orthogonal test or response surface method to optimize fermentation conditions (Zhang *et al*., 2022).

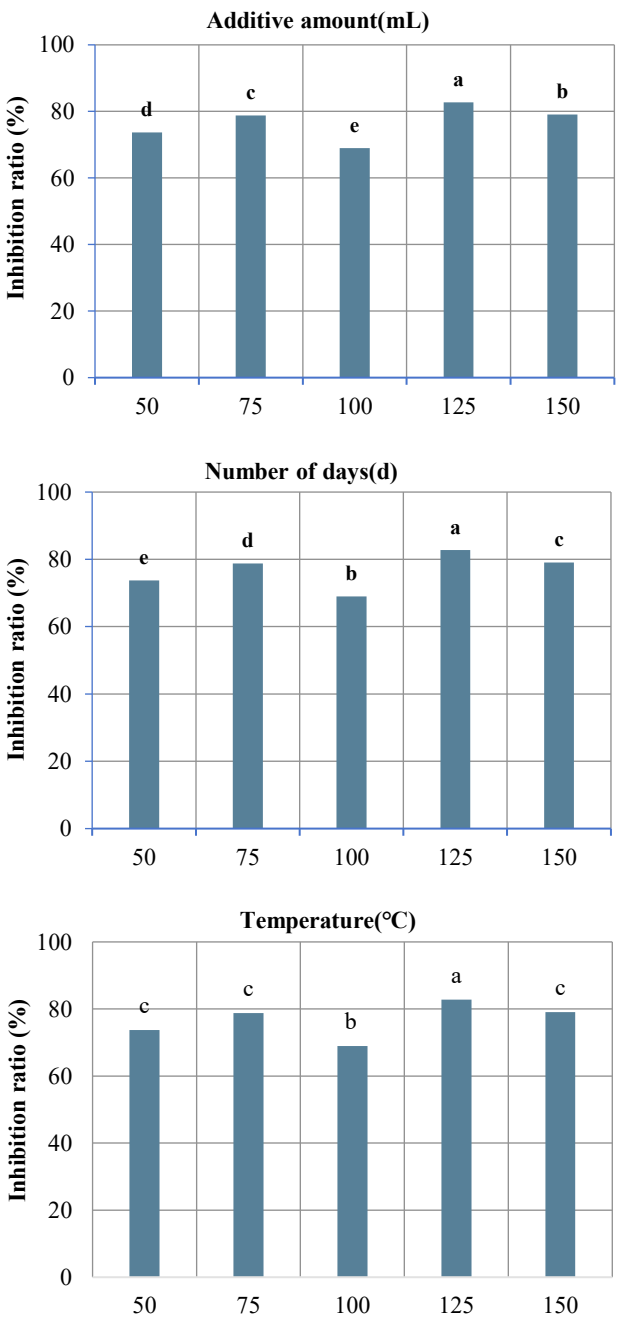

Fig. 8. Bacteriostatic rate of fermentation liquid after different loading amount, fermentation time and temperature treatment

At the beginning of the experiment, a biocontrol fungus F11 strain was screened and identified as *Talaromyces sayulitensis*. In this experiment, the fermentation medium composition, component addition amount and fermentation conditions of F11 strain were optimized by single factor method, orthogonal test method and response surface method. The results showed that the inhibition rate was the highest when the optimum carbon and nitrogen sources and inorganic salts were corn gluten meal, diammonium phosphate and $CaCO_3$, and the optimum addition levels were 24.33 $g \cdot L^{-1}$, 2.56 $g \cdot L^{-1}$ and 1.52 $g \cdot L^{-1}$, respectively. It further illustrates the biocontrol potential of F11 strain, provides a good advantage for the future production of better biocontrol agents, and can better play the role of biocontrol in agricultural production.

Acknowledgements

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